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REVIEW

Hydrogenosomes: convergent adaptations of mitochondria to anaerobic environments**

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Summary

Hydrogenosomes are membrane-bound organelles that compartmentalise the final steps of energy metabolism in a number of anaerobic eukaryotes. They produce hydrogen and ATP. Here we will review the data, which are relevant for the questions: how did the hydrogenosomes originate, and what was their ancestor? Notably, there is strong evidence that hydrogenosomes evolved several times as adaptations to anaerobic environments. Most likely, hydrogenosomes and mitochondria share a common ancestor, but an unequivocal proof for this hypothesis is difficult because hydrogenosomes lack an organelle genome – with one remarkable exception (*Nyctotherus ovalis*). In particular, the diversity of extant hydrogenosomes hampers a straightforward analysis of their origins. Nevertheless, it is conceivable to postulate that the common ancestor of mitochondria and hydrogenosomes was a facultative anaerobic organelle that participated in the early radiation of unicellular eukaryotes. Consequently, it is reasonable to assume that both, hydrogenosomes and mitochondria are evolutionary adaptations to anaerobic or aerobic environments, respectively.

Key words: hydrogenosomes, mitochondria, evolution, eukaryotes, anaerobes

Introduction

Life on earth evolved under anaerobic conditions until oxygenic photosynthesis provided the basis for the evolution of aerobic organisms (Schopf and Klein, 1992). Our everyday's experience suggests that this transition from a reducing to an oxidising atmosphere was a revolution that triggered the evolution of a biosphere totally dominated by aerobic organisms. This view, however, does not withstand a deeper analysis. A wealth of anoxic and microaerobic environments has persisted from the dawn of evolution, still providing niches for the most divergent and complex anaerobic microbial communities. Until today freshwater, and oceanic sediments, continental aquifers, and even porous rocks host a bewildering anaerobic microbiota (Ghiorse, 1997; Whitman et al., 1998). These environments represent the largest ecosystems world-wide, and they still play a crucial role in the global nutrient cycles. Due to the fractal structure of these anaerobic niches (Mandelbrot, 1982), microorganisms predominate in these anaerobic communities (Fenchel and Finlay, 1995). Also, the gastro-intestinal tracts of the various animals – regardless whether in termites, cockroaches, cattle or man – provide unfathomed niches for extraordinary complex and numerous anaerobic communities (Hungate, 1966; Sav-

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age, 1977; Miller and Wolin, 1986; Cruden and Markovetz, 1987; Hobson, 1988; Hackstein and Stumm, 1994; Hackstein and van Alen, 1996; Brune and Friedrich, 2000; Cazemier et al., 1997; van Hoek et al., 1998; Brauman et al., 2001). Thus, in contrast to our everyday's experience, the biosphere still provides a wealth of anoxic niches that are populated by myriads of anaerobic microorganisms. A number of the eukaryotic microorganisms evolved highly specialised adaptations of their terminal energy metabolism: "anaerobic" mitochondria and hydrogenosomes.

Adaptations to anaerobic environments

Organisms that can persist in anaerobic communities are highly adapted to life without oxygen. Anaerobic prokaryotes, for example, evolved a wide variety of alternative, anaerobic respiration processes: instead of oxygen, certain prokaryotes can use electron acceptors such as nitrate, sulphate, carbonate, iron, or even protons (Castresana and Moreira, 1999; Lengeler et al., 1999). Alternatively, and sometimes additionally, many prokaryotes invented a broad spectrum of fermentation pathways in order to maintain a proper oxidation-reduction balance under anaerobic conditions (Lengeler et al., 1999). Eukaryotes, on the other hand, rely nearly exclusively on glycolysis for their survival under anaerobic conditions. Only a rather limited number of eukaryotes evolved alternative, anaerobic respiratory pathways (Tielens, 1994; Embley and Martin, 1998; Grieshaber and Völkel, 1998; Tielens and van Hellemond, 1998). It might be concluded that evolution clearly favoured those eukaryotes that succeeded to maintain their redox-balance under anaerobic conditions by fermentation. We will discuss whether this is due to Dollo's law (see below). In fact, most anaerobic eukaryotes degrade glucose (in the cytoplasm) to ethanol, lactate or other partially reduced compounds that could yield substantial amounts of ATP if oxidised in the (mitochondrial) TCA cycle. In addition, an organellar electron transport chain that is capable of using either oxygen or an alternative electron acceptor could further improve the energy yield by oxidising the reduction equivalents. The glycolytic Embden-Meyerhoff pathway yields only two mol ATP per mol glucose when it is metabolised to pyruvate; organic electron acceptors and reduced cofactors, such as NADH, that are generated during glycolysis are "wasted" under anaerobic conditions. In other words, under anaerobic conditions, the reduction equivalents formed during the catabolic degradation of glucose are transferred to endogenous acceptors that give rise to the various fermentation products with no (or only a rather limited) additional yield of energy. In the presence of oxygen, the mitochondrial electron transport chain of (aerobic and facultative anaerobic) eukaryotes allows the complete oxidation of glucose in the Embden-Meyerhoff pathway and the TCA cycle with a net-gain of approximately 30 to 32 mol ATP per 1 mol glucose (Nelson and Cox, 2000).

Organisms without mitochondria: *"archaezoa"*?

Trivially, the "normal" mitochondrial electron transport chain cannot be used for the generation of ATP under anaerobic conditions, it requires oxygen as terminal electron acceptor. Consequently, mitochondria can fulfil their energy conservation function only in a rather limited way (if at all) under anaerobic conditions. Therefore, it is not surprising that a number of present day anaerobic eukaryotes lack such "useless" mitochondria (*type I anaerobic eukaryotes*). The absence of mitochondria in these organisms, however, can be interpreted in different ways, either as a *primitive* or a *derived* character. Until recently, anaerobic eukaryotes without mitochondria had been interpreted as "primitive" organisms; consequently, they were called "archaezoa" (Cavalier-Smith, 1993). These organisms were supposed to be relics of ancestral, primitive eukaryotes that evolved in the dawn of evolution before the advent of atmospheric oxygen, and of mitochondria (Cavalier-Smith, 1993; Margulis, 1993; Fenchel and Finlay, 1995).

Currently, the assumption that the extant amitochondriate eukaryotes are "primitive" is no longer favoured. Rather, amitochondriate eukaryotes such as *Giardia*, *Entamoeba*, and the various microsporidia are regarded as highly derived eukaryotes that evolved by a differential loss of their aerobic metabolism in adaptation to a parasitic, anaerobic life-style (Fig. 1; Roger, 1999; Baldauf et al., 2000; van de Peer et al., 2000). Persuasive evidence has been presented that these anaerobic, amitochondrial eukaryotes possess mitochondrial-type chaperonines and a number of enzymes that are likely to be relics of an ancestral mitochondrion (see for example Roger et al., 1998; Roger, 1999). Since lateral gene transfer between an aerobic mitochondriate and an anaerobic amitochondriate eukaryote is very unlikely, the presence of "mitochondrial" proteins in amitochondriate anaerobes is most easily explained as the result of a loss of mitochondria. These proteins are, of course, encoded by nuclear genes, as the vast majority of all mitochondrial proteins (see below). Notably, in *Entamoeba*, a vestigial organelle, called "mitosome" or "crypton" – has been identified that might represent a relic mitochondrion (Mai et al., 1999; Tovar et al., 1999). Thus, it is plausible, that, at least in certain

amitochondriate anaerobic protists, the adaptation of eukaryotic microorganisms to anoxic niches implicated the loss of mitochondria (*type I anaerobes*; Fig. 1; Martin and Müller, 1998). Moreover, since none of the currently known amitochondriate eukaryotes is completely devoid of "mitochondrial" genes, it might be questioned as to whether amitochondriate "archaezoa" ever existed. This phenomenon might also explain why most of the extant type I anaerobic eukaryotes rely on (cytoplasmic) glycolysis: the mitochondrial pathways have been lost once, and they will never be acquired a second time as predicted by Dollo's law (Dollo, 1893). Thus Dollo's law precludes the evolution of alternative, anaerobic electron transport chains, if the ancestral mitochondria have been lost once.

Anaerobic mitochondria

Of course, an adaptation of eukaryotes to anaerobic environments does not necessarily imply a loss of mitochondria. For example, the yeast *Saccharomyces cerevisiae* is able to grow under strictly anaerobic conditions, without loss of its mitochondria. However, cultivation of *S. cerevisiae* under anaerobic conditions does not allow the generation of ATP by its mitochondria **(**Bakker et al., 2001). Since the mitochondria of *S. cerevisiae* did not retain a mitochondrial complex I, ATP synthesis under anaerobic conditions solely relies on the glycolytic fermentation of glucose to ethanol. In the presence of oxygen, the fermentation pathway is inhibited and the yeast's metabolism is switched to aerobic respiration with its much higher energy yield ("Pasteur-effect"). Also, certain multicellular organisms such as parasitic helminths, freshwater snails, mussels, lugworms, and certain marine invertebrates, which become regularly exposed to more or less extended periods of anaerobiosis, evolved adaptations to cope with anoxic challenges. These animals evolved substantial modifications of their mitochondrial metabolism in adaptation to anoxic or microaerobic environments (Tielens, 1994; Grieshaber and Völkel, 1998; Tielens and van Hellemond, 1998). Some of these organisms evolved a peculiar variant of anaerobic respiration, "malate dismutation", by which endogenous fumarate is reduced to succinate by the enzyme fumarate reductase. In these organisms, fumarate serves as an electron sink. This process requires adaptations of the mitochondrial electron transport chain, i.e., rhodoquinone instead of ubiquinone as electron-carrier (Tielens and van Hellemond, 1998). However, the fumarate "respiration" allows functioning of mitochondrial complex I, i.e., the generation of a proton gradient, also under anoxic conditions. Since the generation of a proton-motive force (PMF) by mitochondrial complex I can be used for the generation of additional ATP, one might conclude that this adaptation is one of the major reasons for the maintenance of the mitochondrial compartment in multicellular anaerobic eukaryotes.

Type II anaerobes: organisms that host *"hydrogenosomes"*

In certain anaerobic protists and some anaerobic chytridiomycete fungi the adaptation to anoxic niches was accompanied by the evolution of "hydrogenosomes" (*"type II anaerobes"*, Müller, 1993, 1998; Martin and Müller, 1998; Fig. 1) These hydrogenosomes are membrane-bound organelles that measure approximately 1–2 micrometer. They compartmentalise the terminal reactions of the anaerobic cellular energy metabolism and produce hydrogen and ATP. Characteristically, hydrogenosomes import pyruvate that is oxidatively decarboxylated to acetyl-CoA by the action of a pyruvate:ferredoxin oxidoreductase (PFO). An acetate:succinylCoA transferase (ASCT) and a succinate thiokinase (STK) mediate the formation of acetate and ATP, similar to the situation in the "primitive" mitochondria of certain trypanosomes (Fig. 2; Müller, 1993, 1998; van Hellmond et al., 1998; Hackstein et al., 1999). The reduction equivalents that are formed in the decarboxylation of pyruvate are used by a hydrogenase to reduce protons under the formation of molecular hydrogen.

Hydrogenosomes do not co-exist with mitochondria, and, notably, they have neither been detected in multicellular organisms nor in facultative anaerobes that face extended periods of aerobiosis during their life cycles (Roger, 1999). They are found exclusively in anaerobic or microaerophilic unicellular eukaryotes. Since hydrogenosomes compartmentalise terminal reactions of the eukaryotic cellular energy metabolism, they can be regarded as a kind of "anaerobic mitochondria" (Embley et al., 1997; Hackstein et al., 1999; Rotte et al., 2000). In their hypothesis for the origin of the eukaryotic cell, Martin and Müller (1998) suggested that hydrogenosomes and mitochondria are just alternative issues of the same symbiont that evolved from the primordial syntrophic association of prokaryotes that eventually gave rise to the eukaryotic cell. They postulated that mitochondria and hydrogenosomes evolved by differential loss of the aerobic and anaerobic pathways, respectively, in aerobic and anaerobic eukaryotes. Although this hypothesis is very persuasive, its validation is complicated by the fact that hydrogenosomes, in contrast to mitochondria, did not retain a genome that could prove its mitochondrial descent. With one remarkable exception that will be discussed below (i.e., *Nyctotherus ovalis*), the ancestral symbiont lost its genome com-

pletely during its evolution from symbiont to hydrogenosome (Fig. 3; Palmer, 1997). Notably, also mitochondria lost most of their genes: only a minimal fraction of the symbiont's genome has been retained. The vast majority of the mitochondrial genes have been transferred to the nucleus, with the consequence that most of the mitochondrial proteins are synthesised in the cytoplasm and eventually imported into the mitochondria. In the case of the evolution of most hydrogenosomes, the symbiont's genome has been lost completely with the consequence that all hydrogenosomal proteins are now encoded by nuclear genes, synthesised in the cytoplasm and imported into the hydrogenosome. Since the monophyletic origin of mitochondria could only be validated by an analysis of their complete residual genomes (Gray et al., 1999), the proof for a mitochondrial origin of hydrogenosomes will be much more complicated (Anderson and Kurland, 1999).

Hydrogenosomes are not the same and evolved several times

Hydrogenosomes have been discovered nearly 30 years ago in the parasitic parabasalid flagellates *Trichomonas vaginalis* and *Tritrichomonas foetus* (for review see Müller, 1993). Their metabolism and structure has been studied intensively (Fig. 2, 4; Müller, 1993, 1998; Benchimol et al., 1996a, b). Subsequently, hydrogenosomes have been found in various, phylogenetically rather unrelated eukaryotes such as, for example, the amoebo-flagellate *Psalteriomonas lanterna*, the ciliates *Trimyema compressum*, *Pla-*

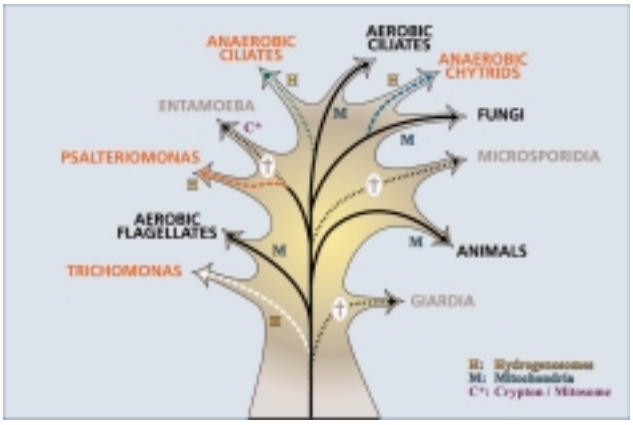

Fig. 1. Cartoon displaying the phylogenetic relationships between aerobic and anaerobic protists (based on a variety of molecular data) together with a tentative evolutionary tree of mitochondria and hydrogenosomes. There is evidence that mitochondria have been lost in organisms such as Microsporidia, *Giardia*, and *Entamoeba*. The latter organism has retained a vestigial organelle, named "crypton" or "mitosome" of unknown function (Mai et al., 1999; Tovar et al., 1999). Anaerobic organisms such as *Trichomonas*, *Psalteriomonas* and the anaerobic ciliates and chytrids evolved hydrogenosomes (Müller, 1993), whereas the aerobic protists and the multicellular animals adapted to aerobic environments retaining aerobic descendants of the ancestral, facultative anaerobic mitochondrion (Martin and Müller, 1998).

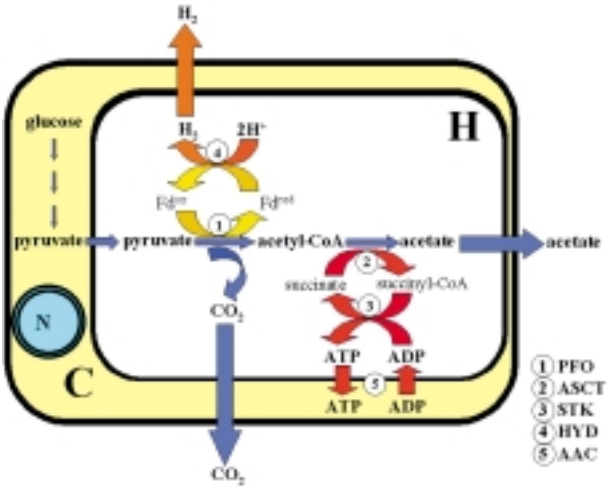

Fig. 2. Metabolic scheme of a generalised anaerobic protist with a hydrogenosome ("type II anaerobe"; Müller, 1993, 1998). Pyruvate is formed in the cytoplasm (C) by glycolysis, imported into the hydrogenosome (H) and metabolised to acetate and CO_2 under formation of H_2. ATP is formed by substrate level phosphorylation by the enzymes acetate succinyl-CoA transferase (ASCT, 2) and succinate thiokinase (STK, 3). ATP is exported by an ADP-ATP carrier (AAC, 5). The electrons resulting from the oxidative decarboxylation of pyruvate are transferred to a ferredoxin by pyruvate:ferredoxin oxidoreductase (PFO, 1) and to protons by a Fe-hydrogenase (HYD, 4). N: nucleus

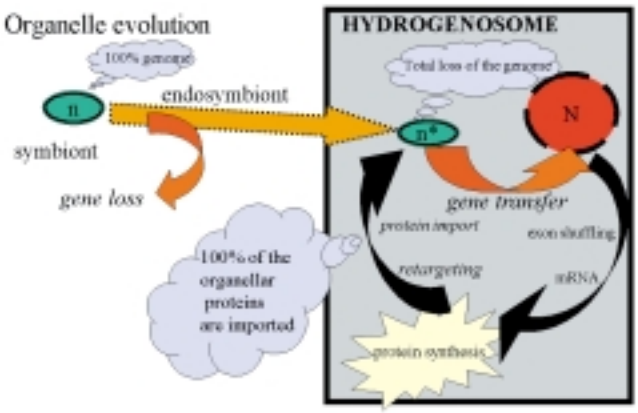

Fig. 3. Cartoon of crucial events in the evolution of hydrogenosomes (Palmer, 1997; Martin and Müller, 1998). The evolution of mitochondria and hydrogenosomes from a free-living, facultative anaerobic prokaryote was accompanied by a massive loss of redundant and superfluous genes; other genes were transferred to the nucleus (Herrmann, 1997; Martin and Herrmann, 1998). In the evolution of hydrogenosomes the organellar genome has been lost completely (with one exception, see text). This implicates that all hydrogenosomal proteins became nuclear encoded, and are now synthesised in the cytoplasm, targeted to the organelle and imported by the hydrogenosomal import machinery.

giopyla nasuta, *Dasytricha ruminantium*, *Nyctotherus ovalis*, and the chytridiomycete fungi *Neocallimastix* spec. and *Piromyces spec.* (Fig. 1; Vogels et al., 1980; Yarlett et al., 1981, 1983; van Bruggen et al., 1983, Zwart et al., 1988; Broers et al., 1990; Gijzen et al., 1991; Marvin-Sikkema et al., 1992, 1993b; Hackstein et al., 1999). Consequently, the questions arose whether (i) all hydrogenosomes are the same and (ii) all of them evolved from mitochondria (Müller, 1993; Coombs and Hackstein, 1995; Embley et al., 1997). Since the striking metabolic differences between the various hydrogenosomes have been subject to a recent review (Hackstein et al., 1999), we will focus here on evolutionary issues and the ultrastructure of the various hydrogenosomes.

Trichomonas vaginalis/Tritrichomonas foetus

As already mentioned, hydrogenosomes have been first discovered in *Trichomonas vaginalis* and its relative *Tritrichomonas foetus* (Müller, 1993). The phylogenetic position of the host is still subject to discussions (Roger, 1999; Philippe and Germot, 2000), but it seems likely that it is related to the primitive, giant polymastigote flagellates from the hindgut of termites **(**Ohkuma et al., 2000**)**. Notably, *T. vaginalis* has no aerobic relatives that could host "normal" mitochondria. Electron microscopy revealed that the hydrogenosomes of the trichomonads are more or less spherical, about 1 µm in size, and surrounded by a double membrane (Fig. 4). The matrix of these organelles does not contain

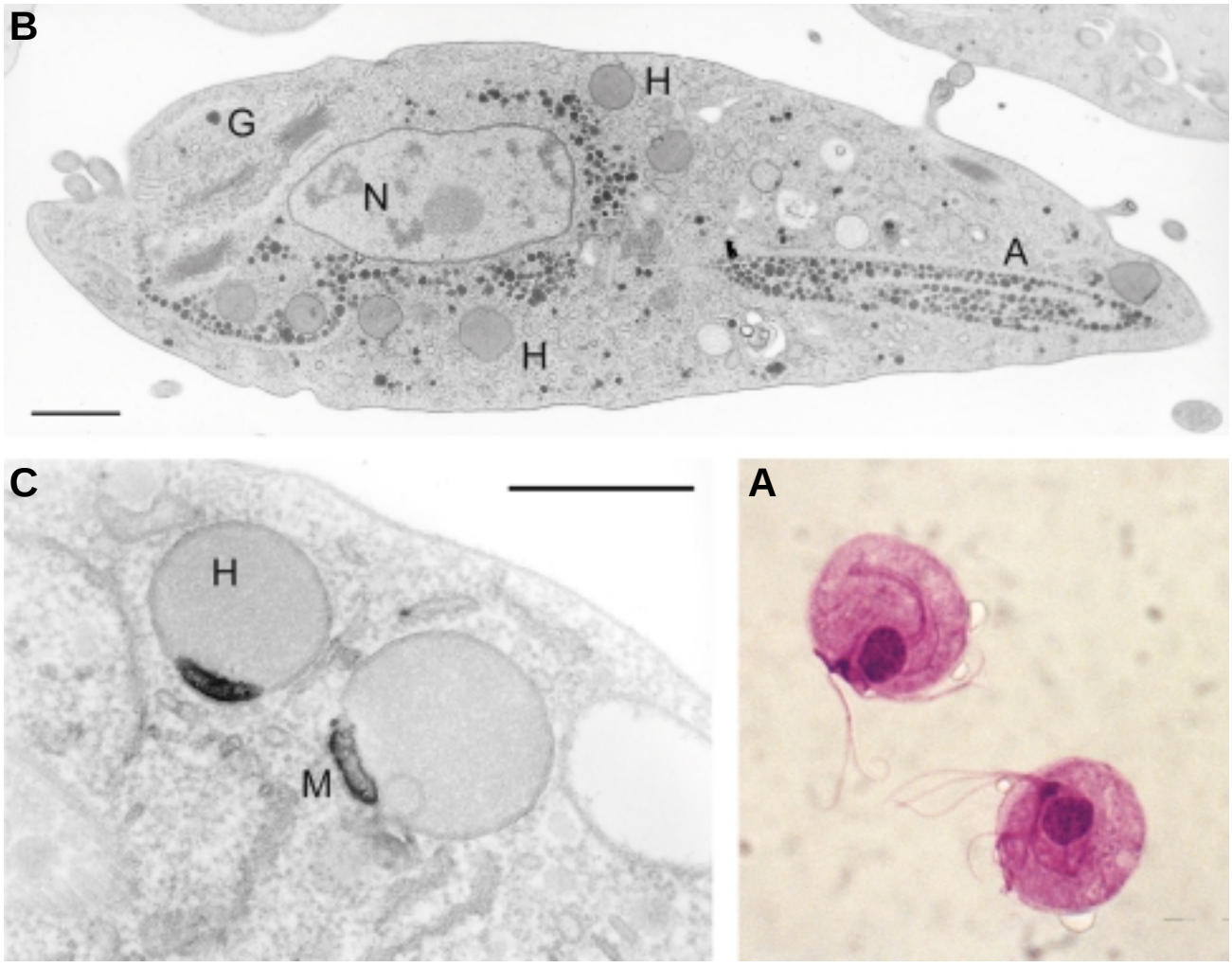

Fig. 4. *Trichomonas:* the "golden standard" for studies on hydrogenosomes (Müller, 1993, 1998). **(A)** *Trichomonas vaginalis*, light microscopical picture of eosin-stained cells; natural size approximately 10 by 45 µm (courtesy of H. Aspöck, Vienna; c.f. Aspöck, 1994). **(B)** Electron micrograph of *Tritrichomonas foetus*, seven hydrogenosomes (H) can be identified in the cytoplasm (N: nucleus; G: Golgi apparatus; A: axostyl). **(C)** A higher magnification reveals that a double membrane surrounds the hydrogenosomes. (M: marginal plate). (B) and (C) were kindly provided by M. Benchimol, Rio de Janeiro (c.f. Benchimol et al., 1996a, b). Bar in (B) and (C) 1 micrometer.

particles that can be interpreted as ribosomes. There is also no evidence for the presence of mitochondria-type cristae or tubuli, and all attempts to identify a hydrogenosomal genome by biochemical or cytochemical means had negative results (Clemens and Johnson, 2000). Consequently, all of the approximately 200 proteins identified in the hydrogenosomes of *T. vaginalis* (Heinze, 2001) should be encoded by nuclear genes, synthesised in the cytoplasm and imported into the hydrogenosome posttranslationally (Fig. 3). Phylogenetic analysis of these genes revealed the presence of "mitochondrial-type" chaperonines, and the presence of a member of the mitochondrial transporter family with unknown function (Dyall and Johnson, 2000; Dyall et al., 2000). However, evidence in favour of a mitochondrial ancestry of all of the hydrogenosomal genes is still lacking. For example, one of the hydrogenosomal key enzymes, pyruvate:ferredoxin oxidoreductase (PFO) is definitively not of α-proteobacterial origin, just as the Fe-hydrogenases that have been identified in the hydrogenosomes of *T. vaginalis* (Payne et al., 1993; Horner et al., 1999, 2000). Thus, there is ample evidence that the hydrogenosomes of *T. vaginalis* share a common ancestry with mitochondria; however, an unequivocal, straightforward proof for this relationship is still lacking.

Psalteriomonas lanterna

The anaerobic amoebo-flagellate *Psalteriomonas lanterna* is a primitive representative of the *Vahlkamphiidae (Percolozoa)*, a taxon that consists predominantly of aerobic, mitochondriate species (Broers et al., 1990; Fig. 5, 6). In *P. lanterna*, however, a unique type of hydrogenosomes could be identified with the aid of a histochemical hydrogenase assay (Zwart et al., 1988; Broers et al., 1990). Disc-like to sausage-shaped organelles are stacked to form a globular, giant hydrogenosome that is clearly visible even at low magnification (Fig. 5; Broers et al., 1990). As in *Trichomonas spp.*, the individual organelles are surrounded by a double membrane, possess no internal membranous differentiations, exhibit no structures that might be interpreted as ribosomes or organellar nucleoids, and the

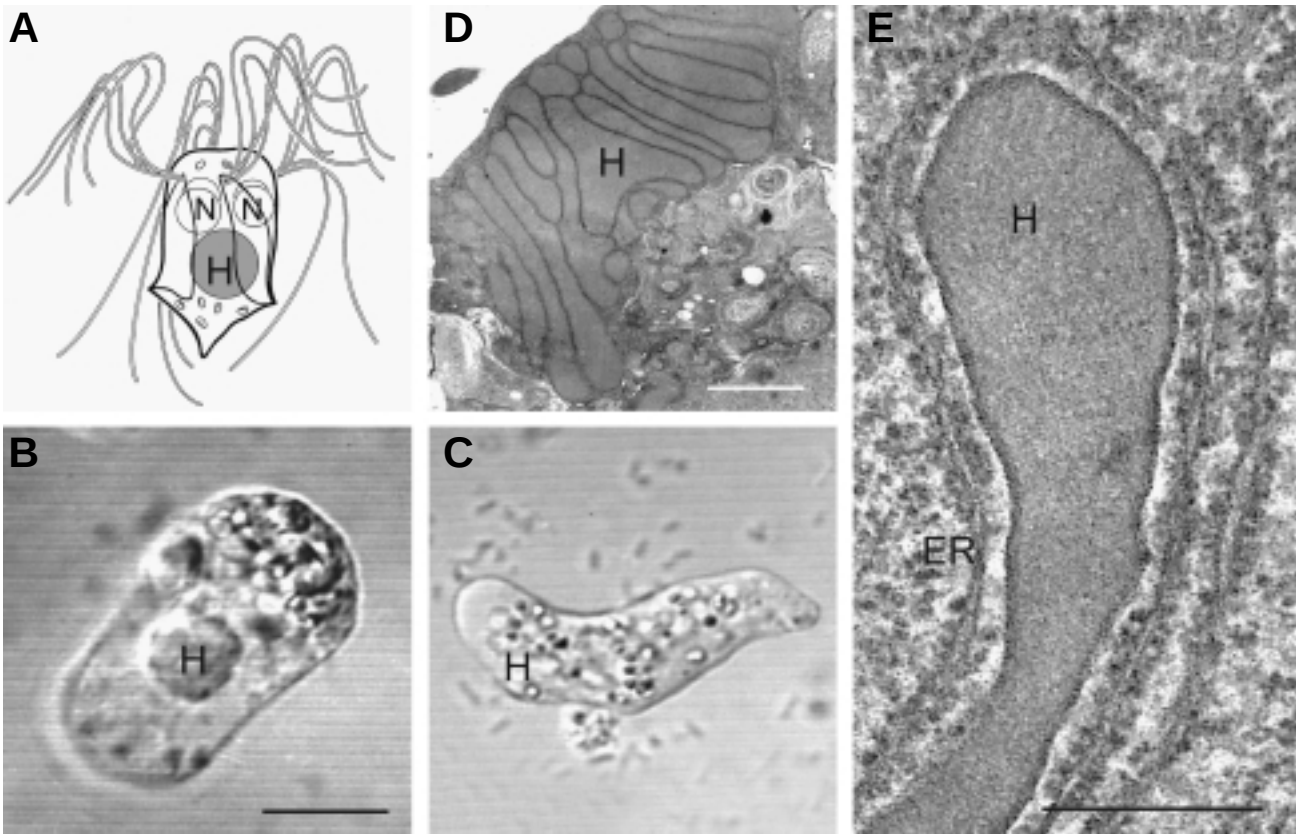

Fig. 5. *Psalteriomonas lanterna* (Broers et al., 1990). This amitochondriate amoeboflagellate possesses 4 nuclei (N) and 16 flagella when it thrives in the flagellate stage **(A, B)**, and 1–2 nuclei when it exists in the amoeba stage **(C)**. Its hydrogenosomes form a voluminous complex that consists of many individual, stacked hydrogenosomes H in **(A)**, **(B)**, **(C)**, and **(D)**. In the periphery of the flagellate cell, individual hydrogenosomes are found to be surrounded by 1–2 cisterns of rough endoplasmatic reticulum (ER in **E**). Bars for **(A)**, **(B)**, **(C)**: 10 µm; for **(D)** and **(E)**: 1 µm. The help of G. Kreimer, Cologne, with the CLS microscope (B, C) is gratefully acknowledged.

staining with DAPI or ethidium bromide provided no evidence for the presence of DNA. In the periphery of the cells, individual, dividing hydrogenosomes can be found, often in close association with the endoplasmatic reticulum (ER). Characteristically, the peripheral, individual hydrogenosomes are surrounded by 1–2 cisterns of rough ER (Fig. 5).

Little is known about the biochemistry, physiology and phylogeny of these hydrogenosomes. A putative ferredoxin has been identified. It resembles the ferredoxin of *T. vaginalis* (Brul et al., 1994). However, phylogenetic analysis of the 18S rRNA genes does not support a close relationship between *P. lanterna* and *T. vaginalis* (Fig. 6) and seems to exclude a *recent* common origin of their hydrogenosomes. Thus, the data that favour a mitochondrial ancestry of the hydrogenosomes of *P. lanterna* are rather circumstantial.

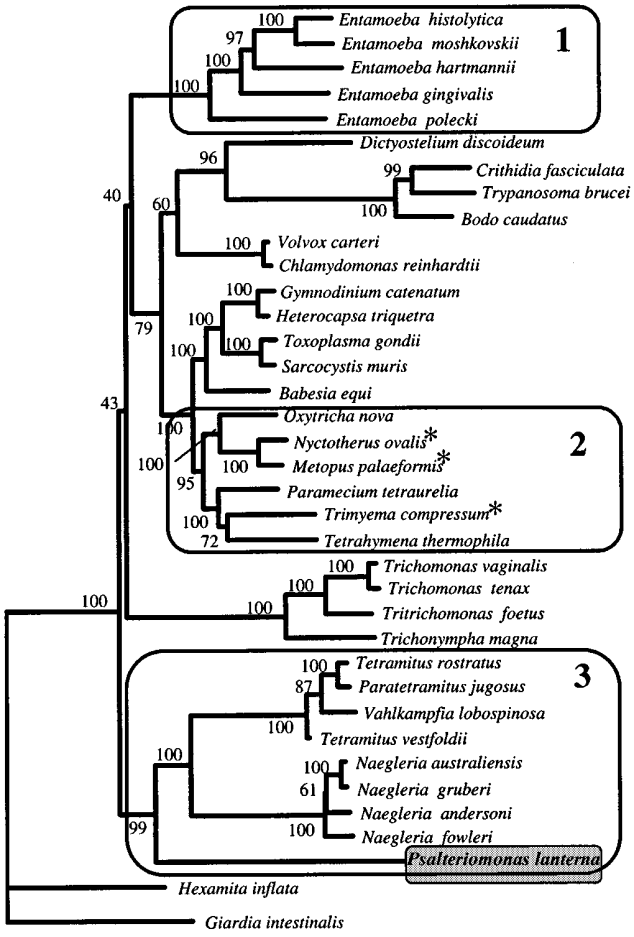

Fig. 6. Neighbour-Joining tree ("NJ"; Saitou and Nei, 1987) of the 18S rRNA genes of a number of aerobic and anaerobic protists illustrating the phylogenetic position of *Psalteriomonas lanterna* (Acc. Nr. AF 420005). Whereas the group of the various species of *Entamoeba* consists exclusively of anaerobic organisms (box 1), box 2 combines both aerobic and anaerobic (*) ciliates. Box 3 indicates the closest relatives of *Psalteriomonas lanterna*, the *Percolozoa* and *Vahlkamphiidae*, that represent aerobic, mitochondriate species.

Neocallimastix sp. L2/*Piromyces* sp. E2

The anaerobic chytrids *Neocallimastix* and *Piromyces* are biochemically, physiologically and phylogenetically related to aerobic yeasts and fungi (see Akhmanova et al., 1998b, 1999 for discussion; Brookman et al., 2000). They thrive in the gastro-intestinal tract of large herbivorous vertebrates (Fig. 7). Their hydrogenosomes have been studied extensively at the physiological and biochemical level (Marvin-Sikkema et al., 1992, 1993a, b, 1994; van der Giezen et al., 1997a, b, 1998; Akhmanova et al., 1998b, 1999; Voncken, 2001). All these studies revealed that the hydrogenosomes of chytrids differ from all other hydrogenosomes studied so far (Hackstein et al., 1999), in particular with respect to their ultrastructure (Fig. 8). After classical glutaraldehyde fixation and freeze-substitution the hydrogenosomes of chytrids are bounded by a single membrane that surrounds one (or several) internal vesicles (Fig. 8; Marvin-Sikkema et al., 1992, 1993a, b; van der Giezen et al., 1997a). Van der Giezen et al. (1997a) interpreted these pictures in favour of double-walled organelles, just as Benchimol et al. (1997) who used a different species of chytrid, different fixation and staining methods. However, the electron micrographs displayed in both publications are not substantially different from those published earlier by Marvin-Sikkema et al. (1992, 1993a, b; see also Fig. 8). At all fixation conditions, and irrespective whether the hydrogenosomes were present in free-swimming, flagellated zoospores or a vegetative mycelium, internal membranous structures can be observed. Frequently,

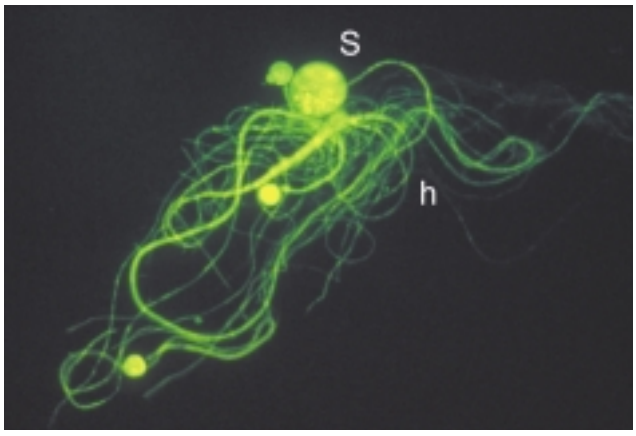

Fig. 7. Epifluorescence micrograph of *Piromyces* sp. E2, an anaerobic chytridiomycete fungus, isolated from the faeces of an Indian elephant. Magnification about ×400. The organism was vitally stained with a solution of rhodamine 123. h: hyphae; S: sporangium.

the “inner” and “outer” membranes are closely opposed at parts of the organelle resembling a classical “double” membrane. However, these structures do not look like mitochondrial cristae or tubuli. Rather the organelles resemble human mitochondria affected by hereditary mitochondrial diseases (Smeitink et al., 1989; Huizing et al., 1997; Frey and Mannella, 2000).

The molecular data clearly indicate the mitochondrial ancestry of a number of proteins of these anaerobic chytrids. However, several enzymes of mitochondrial origin were localised in the cytoplasm and not in the hydrogenosomes (Akhmanova et al., 1998b; Hackstein et al., 1999). Phylogenetic analysis of a gene encoding a putative organellar chaparonine (HSP 60) clearly clusters with its mitochondrial homologues from aerobic fungi, and a hydrogenosomal ADP/ATP transporter (AAC) has been identified that exhibits all characteristics of a fungal mitochondrial adenine nucleotide transporter (Voncken, 2001). This hydrogenosomal transporter strongly supports a common ancestry of chytrid hydrogenosomes and fungal mitochondria –notwithstanding their highly different morphology and the absence of a hydrogenosomal genome.

Nyctotherus ovalis

In at least 8 of the 22 orders of ciliates as classified by Corliss (1979), anaerobic or microaerophilic species evolved that can live permanently in the (nearly) complete absence of oxygen. Three more orders, i.e. the *Karyolectides*, *Hypotrichs* and *Prostomatids*, encompass a number of facultative anaerobes (Fenchel and Finlay, 1995). All ciliates possess energy-generating organelles – either mitochondria (the aerobic ones) or hydrogenosomes (the anaerobic ones). *Nytotherus ovalis* belongs to a monophyletic group of anaerobic heterotrichous ciliates (classified also as *Clevelandellids* and *Armophorids*, Small and Lynn, 1981) that thrive in the intestinal tracts of millipedes, cockroaches and frogs, but also in the freshwater and marine sediments (Fig. 9;

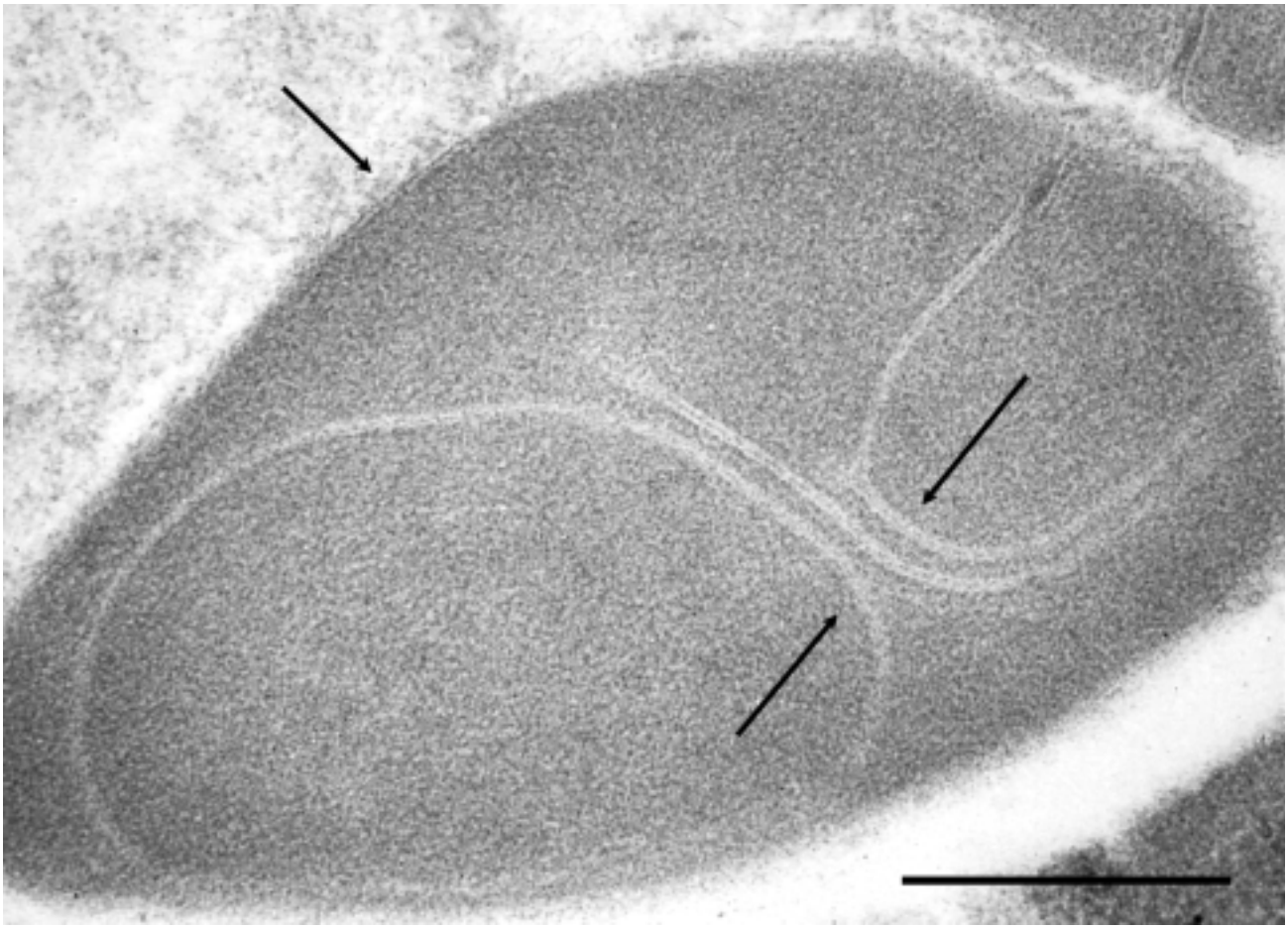

Fig. 8. Electron micrograph of a hydrogenosome of the anaerobic chytrid *Neocallimastix* sp. L2, isolated from the faeces of a llama. This type of hydrogenosomes has a morphology very different from that of *Trichomonas* sp. or *Nyctotherus ovalis.* Arrows indicate the internal membranes and vesicles. Bar 0.5 micrometer

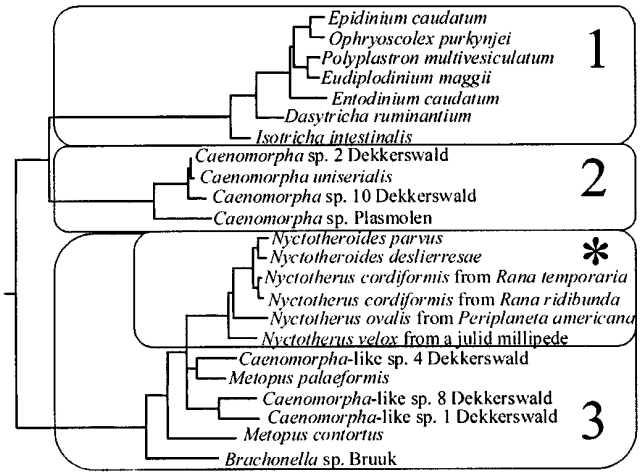

Fig. 9. Phylogenetic relationships among anaerobic heterotrichous (2, 3) and rumen ciliates (1). STAR decomposition analysis of the 18S rRNA genes (MOLPHI; Adachi and Hasegawa, 1996). The taxa analysed here consist exclusively of anaerobic ciliates that (most likely) possess hydrogenosomes. They belong to three different groups, which are consistently identified, regardless of the phylogenetic methods (Neighbour Joining; Saitou and Nei, 1987; PUZZLE; Strimmer and von Haeseler, 1996) that are used to calculate the phylogenetic trees. All other branching in the tree has a low statistical support and is sensitive to the sampling of species. The * indicates intestinal ciliates from frogs, millipedes and cockroaches. All other species in 2 and 3 are free-living (c.f. van Hoek et al., 1998, 2000b), all species displayed in box 1 are living in the rumen of ruminants.

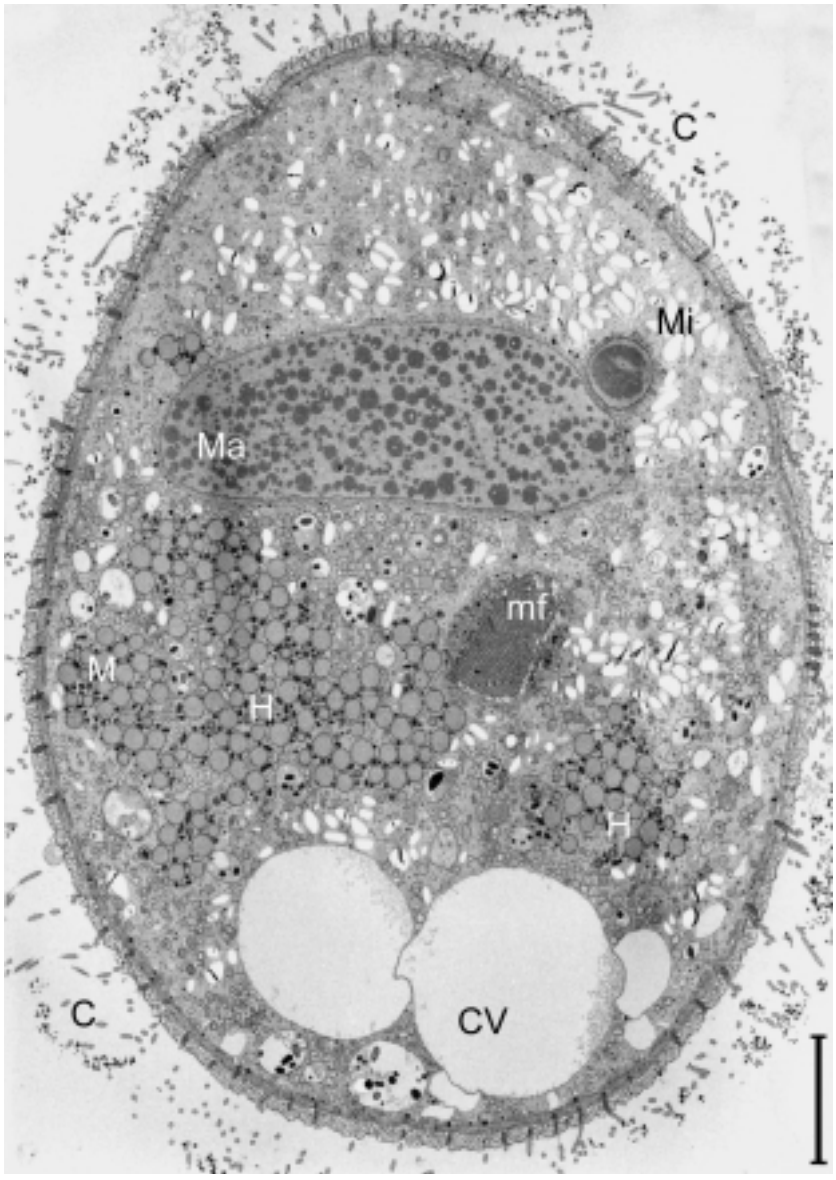

Fig. 10. EM picture of *Nyctotherus ovalis*, $KMnO_4$ fixation. Ma: macronucleus; Mi: micronucleus; H: hydrogenosomes; M: methanogenic endosymbionts (dark dots); CV: contractive vacuole; mf: mouth field; C: cilia; Bar 10 µm. (c.f. Akhmanova et al., 1998a).

van Hoek et al., 1998, 1999, 2000b). All these anaerobic heterotrichs possess hydrogenosomes, in many cases only indirectly identified by the presence of methanogenic endosymbionts (Fenchel and Finlay, 1995; van Hoek et al., 2000b). In those species studied in more detail by electron microscopy, the methanogenic archaeal endosymbionts are found in close association with mitochondria-like organelles (Fig. 10, 11). Because of the mitochondria-like morphology, e.g. the presence of mitochondria-like cristae and putative 70S ribosomes (Fig. 11), it was very suggestive to look for a hydrogenosomal genome, although hydrogenosomes were commonly assumed to lack genomes (Palmer, 1997). Notably, we were able to identify a genome in the "mitochondrial" fraction of homogenates of *N. ovalis* (Fig.12). Comparison with the mitochondrial DNA from *Tetrahymena thermophila* suggests that the hydrogenosomal genome might encompass some 40 kb. Genes encoding small subunits of a mitochondrial-type rRNA have been isolated from a number of *N. ovalis* subspecies and their free-living relatives (Fig. 13). These genes are heavily transcribed and Southern-blotting revealed that the rRNA genes must be located on a genome that is substantially larger than a single SSU rRNA gene or an extrachromosomal, amplified ribosomal cistron (Akhmanova et al., 1998a; van Hoek et al., 2000a). Phylogenetic analysis unequivocally shows that the SSU rRNA genes of *N. ovalis* hydrogenosomes share a common ancestry with ciliate mitochondria. There is little doubt that the sequencing of the complete hydrogenosomal genome of *N. ovalis*

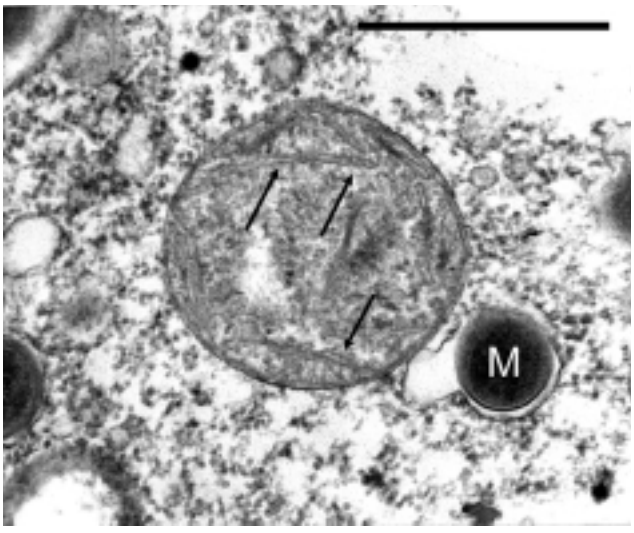

Fig. 11. A hydrogenosome of *Nyctotherus ovalis* at higher magnification (glutaraldehyde/OsO_4 fixation). The inner and outer membrane, crista-like invaginations of the inner membrane (arrows), and putative 70S ribosomes can be identified (black dots in the matrix). M: methanogenic endosymbiont; Bar 1 µm (c.f. Akhmanova et al., 1998a).

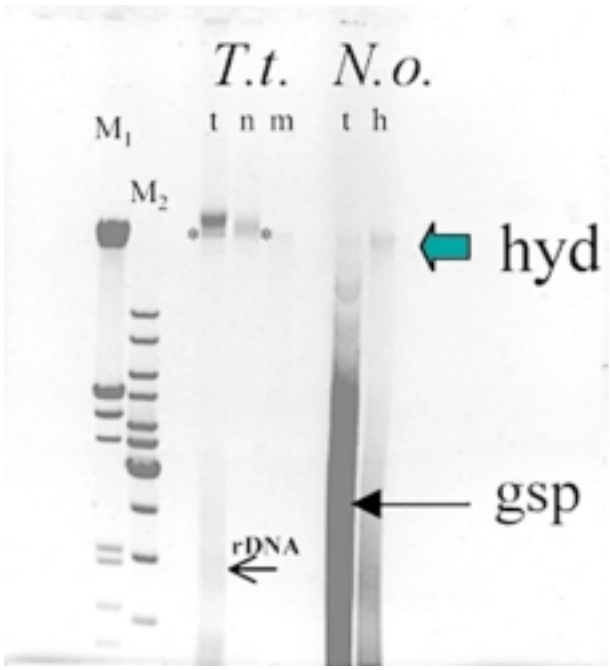

Fig. 12. Nuclear and organellar DNA of *Tetrahymena thermophila (T.t.)* and *Nyctotherus ovalis (N.o.)* after cellular fractionation by differential centrifugation. Agarose gel (0.5%) stained with ethidium bromide. M_1 marker lambda EcoR1/ Hind III, M_2 1 kb ladder.
T.t. lane t: total DNA; lane n: nuclear fraction, consisting predominantly of rDNA and macronuclear DNA; lane m: mitochondrial fraction (*, mitochondrial DNA, >40 kb, also visible in total DNA fraction); *N.o.* lane t: total DNA: it consists nearly exclusively of macronuclear DNA that is present in gene-sized pieces (gsp), predominantly <9 kb; h: hydrogenosomal fraction; hydrogenosomal DNA is indicated by an arrow (hyd). Hydrogenosomal DNA is also clearly visible in the total DNA fraction.

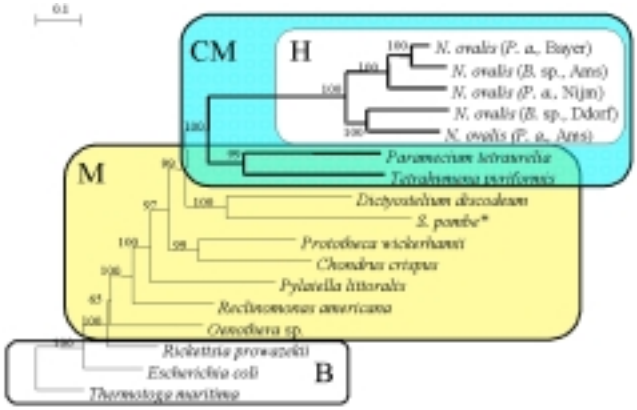

Fig. 13. Phylogenetic tree (Neighbour Joining; Saitou and Nei, 1987) of mitochondrial (M) and hydrogenosomal (H) SSU rRNA genes (c.f. van Hoek et al., 2000a). CM: ciliate mitochondria; B: eubacterial 16S rRNA genes. * : *Schizosaccharomyces pombe*.
Abbreviations: *N. ovalis*: *Nyctotherus ovalis; P.a.: Periplaneta americana; B.* sp.: *Blaberus species*: (cockroach host species). Bayer: Bayer AG, Monheim; Ams: Amsterdam, Artis; Nijm.: Nijmegen, Faculty of Science; Ddorf: Düsseldorf, Germany, Löbbecke Museum (differerent populations of cockroach hosts).

will confirm this conclusion. Thus, both morphology and molecular biology suggest strongly that the hydrogenosomes of anaerobic heterotrichous ciliates are highly specialised mitochondria that produce hydrogen.

Conclusions

Here we have reviewed the available data for the evolution of hydrogenosomes. These data strongly suggest that the hydrogenosomes of the various anaerobic protists evolved repeatedly from "mitochondria" of their closest aerobic or facultative anaerobic ancestors, or from protomitochondria, respectively. This conclusion is supported by both ultrastructural and molecular data. The presence of a hydrogenosomal genome in the anaerobic ciliate *N. ovalis* provides the most straightforward evidence that the hydrogenosomes of anaerobic ciliates share a recent common ancestor with the mitochondria of their aerobic or facultative anaerobic ancestors. It remains to be shown whether the hydrogenosomes in the various ciliates evolved repeatedly and why certain hydrogenosomes retained a genome and others not. Without any doubt, ciliate hydrogenosomes differ substantially from hydrogenosomes of anaerobic chytrids. The phylogenetic analysis of the hydrogenosomal AACs of anaerobic chytrids has shown that these hydrogenosomes evolved from the mitochondria of aerobic yeast and fungi (Voncken, 2001). Proteomics might be a suitable approach to answer the open questions. The origin of hydrogenosomes of *Trichomonas* and *Psalteriomonas* is less clear, due to the lack of data. Notwithstanding, a "mitochondrial"/protomitochondrial origin also of these hydrogenosomes is likely. There are a number of arguments in favour of the hypothesis that ancestral mitochondria were facultative anaerobic organelles, possessing both aerobic and anaerobic metabolic pathways (Martin and Müller, 1998; Rotte et al., 2001). It is obvious that the ancestral mitochondria must have retained their facultative anaerobic nature over extended evolutionary times since they differentiated as "fungal" or "ciliate" mitochondria before adapting to either aerobic or anaerobic niches. Future studies will have to provide the necessary information for an understanding of these adaptations to aerobic and anaerobic environments.

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